Biochemical Basis of Organophosphate and Carbamate Resistance in Asian Citrus Psyllid

SIDDHARTH TIWARI,[1] LUKASZ L. STELINSKI, AND MICHAEL E. ROGERS

Entomology and Nematology Department, Citrus Research and Education Center, University of Florida, 700 Experiment Station Road, Lake Alfred, FL 33850

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ABSTRACT The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is a worldwide pest of citrus, which vectors the putative causal pathogen of huanglongbing. Current management practices warrant continuous monitoring of field populations for insecticide resistance. Baseline activities of acetylcholinesterase (AChE), general esterase, and glutathione *S*-transferase as well as sensitivity of AChE to selected organophosphate and carbamate insecticides were established for a susceptible laboratory strain (Lab) and compared with several field populations of *D. citri* from Florida. The specific activity of AChE in various *D. citri* populations ranged from 0.77 to 1.29 μM min^{-1} mg of protein^{-1}; the Lab strain was characterized by the highest activity. Although reduced AChE sensitivity was observed in the Lab strain compared with field populations, overlap of 95% confidence intervals of I_{50} values (concentration required for 50% AChE activity inhibition) suggests no significant difference in AChE sensitivity among all populations tested for a given insecticide. There was no significant evidence of target site insensitivity in field populations that were exposed to the selected organophosphate and carbamate insecticides tested. The specific activity of general esterase and glutathione *S*-transferase was lowest in the Lab strain and was generally comparable to that of the field populations evaluated. The current data provide a mode-of-action specific baseline for future monitoring of resistance to organophosphate and carbamate insecticides in populations of *D. citri*.

KEY WORDS acetylcholinesterase insensitivity, carbamate, general esterase, glutathione-*S*-transferase, huanglongbing

The Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is an important pest of citrus crops worldwide (Halbert and Manjunath 2004). It is native to Asia and was first discovered in the United States in Florida in 1998 (Halbert and Manjunath 2004). It became a significant economic pest in the United States after the detection of hunaglongbing (HLB) in Florida in 2005 (Halbert 2005). *D. citri* is a vector of phloem-limited bacterial species in the genus *Candidatus* Liberibacter that are presumed to cause HLB (Capoor et al. 1967, Catling 1970, Bové 2006). It is the most destructive disease of citrus worldwide and all citrus cultivars examined to date exhibit some degree of susceptibility (Catling 1970, Bové 2006). The sustainability of $9 billion Florida citrus industry with an annual farm gate value of $1.5 billion (FDACS 2008) is threatened if HLB spread is not controlled.

Given the wide distribution of *D. citri* in Florida and other southern U.S. states in concert with significant capability for dispersal (Boina et al. 2009, Tiwari et al. 2010), chemical control of *D. citri* with several foliar applications of insecticides (6–8/yr) coupled with application of one to two soil systemic insecticides has emerged as a common *D. citri* management practice (Rogers et al. 2008, Srinivasan et al. 2008, Boina et al. 2011, Tiwari et al. 2011a). Such intensive insecticide application schedules and in some cases repeated sequential use of the same insecticide or mode of action has led to the development of varying levels of insecticide resistance throughout Florida (Tiwari et al. 2011a). The majority of recommended insecticides for *D. citri* control fall under two classes of insecticides: organophosphates (OPs) and carbamates.

The OP and carbamate insecticides act by inhibiting one of the key enzymes in the nervous system, acetylcholinesterase (AChE; EC 3.1.1.7) (Zhu and Gao 1999). AChE catalyzes the hydrolysis of the neurotransmitter, acetylcholine, resulting in hyper excitation and death of insects. Reduced sensitivity of AChE as a result of gene mutation causes resistance to OP and carbamate insecticides (Zhu and Gao 1999). Therefore, establishing baseline data on sensitivity of AChE to commonly used OP and carbamate insecticides for a laboratory susceptible (Lab) strain as well as field populations of *D. citri* will allow for monitoring potential shifts in sensitivity of AChE over time. Previously, insecticide resistance in *D. citri* has been re-

[1] Corresponding author, e-mail: stiwari@ufl.edu.

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lated to general esterases (GEST), glutathione S-transferases (GST), and cytochrome P450 monooxygenases (Tiwari et al. 2011a, b, and c); however, target site insensitivity as a mechanism of resistance to OP and carbamate insecticides in *D. citri* required investigation.

The objectives of the current study were to determine: 1) AChE activity and sensitivity to inhibition by selected OP and carbamate insecticides in *D. citri* nymphs and adults for a laboratory susceptible strain and several field-collected populations spanning Florida citrus production, and 2) determine how activity of GEST and GST enzymes may contribute to OP and carbamate resistance in *D. citri*.

Materials and Methods

Insects. A susceptible Lab strain of *D. citri* was reared in a greenhouse at the Citrus Research and Education Center, Lake Alfred, FL. It originated from adults collected in 2000 from citrus in Polk County, FL. This strain has been reared without exposure to pesticides or subsequent input of field-collected *D. citri*. Field-collected populations of *D. citri* were obtained during June through October 2009 from five locations that represent major commercial citrus production areas of Florida: Groveland (Lake County), Fort Pierce (St. Lucie County), La Belle (Hendry County), Lake Alfred (Polk County), and Vero Beach (Indian River County). Management practices in these locations included regular insecticide spray applications for *D. citri* management. At each location, *D. citri* adults (≈8,000–10,000) were collected using sweep nets and aspirated manually into plastic vials and placed into coolers with ice packs for transport to the laboratory. In the laboratory, adults were released into 40 × 40 × 40 cm Plexiglas cages with sleeves containing 3–4 Swingle citrumelo (*X Citroncirus webberi* Ingram and Moore, 2–3 mo-old) plants per cage. *D. citri* nymphs and adults from the Lab strain were used for optimizing the AChE bioassay and determining AChE activity. Field-collected and Lab strain *D. citri* were stored at −20°C before quantifications of AChE specific activity, AChE sensitivity to inhibition by selected OP and carbamate insecticides, and enzymatic assays of GEST and GST.

Insecticides and Reagents. Three OPs [chlorpyrifos (active form: chlorpyrifos-oxon), malathion (active form: malaoxon), dimethoate (active form: omethoate)] and two carbamate [aldicarb (active form: aldicarb-sulfoxide), and carbaryl] insecticides were evaluated to determine baseline susceptibility and AChE sensitivity to inhibition by insecticides. The active forms of insecticides were used in AChE inhibition experiments, where needed, and paraoxon was included as a standard. The technical grades of the above insecticides were purchased either from Chem Service (West Chester, PA) or Sigma–Aldrich (St. Louis, MO). Reagents such as acetylthiocholine iodide (ATChI), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), alpha-naphthyl acetate (α-NA), beta-naphthyl acetate (β-NA), para-nitrophenyl acetate (*p*-NPA), para-nitrophenyl butyrate (*p*-NPB), fast blue B, sodium dodecyl sulfate, α-naphthol, β-naphthol, 1-chloro-2,4-dinitrobenzene (CDNB), reduced glutathione (GSH), bicinchoninic acid, and bovine serum albumin were purchased from Sigma–Aldrich.

AChE Activity Assays. The optimal pH for buffer (0.1 M sodium phosphate) and concentrations of Triton X-100 and Ellman reagents (ATChI and DTNB) were determined for AChE assays following methods developed by Zhu and Gao (1999) and Gao and Zhu (2000) with certain modifications. The AChE source was obtained from adults collected from the Lab strain. Five unsexed adults of mixed age were placed into a 1.5 ml centrifuge tube containing 100 μl of homogenization buffer (ice-cold 0.1 M phosphate buffer + Triton X-100). Adults were homogenized using a hand held homogenizer with plastic pestle (Fisher, Pittsburg, PA). An additional 400 μl of ice-cold homogenization buffer was added and the tube was kept on ice for 10 min giving a ratio of one adult: 100 μl buffer. Various combinations of buffer pH (7.0, 7.5, and 8.0) and Triton X-100 concentration [0.05, 0.1, 0.3, and 0.5% (vol:vol)] were evaluated. The samples were centrifuged at 10,000 × *g* for 15 min at 4°C. After centrifugation, 450 μl of supernatant (enzyme extract) was transferred to clean 1.5 ml centrifuge tubes. Subsequently, it was diluted two-fold by addition of an equal volume of homogenization buffer without Triton X-100 and placed on ice until use in AChE assays.

AChE activity was measured in a 96-well microplate as per the standard procedure described by Ellman et al. (1961) and Zhu et al. (1996) using a microplate reader (SpectraMax 250, Molecular Devices Inc., Sunnyvale, CA) at 405 nm and 25°C. Twenty microliters of the enzyme extract (0.11 insect equivalents) was added to each well in triplicate for each combination of buffer pH and Triton X-100 concentration. Wells with an equal volume of buffer substituted for enzyme source served as controls. To each well, 180 μl of Ellman reagents were added. Ellman reagent was made by mixing 37.5 mM ATChI and 12 mM DTNB in 0.1 M phosphate buffer to give final concentrations of ATChI and DTNB of 0.16 and 0.1 mM, respectively. The plate was read immediately for 30 min at 20 s intervals. Mean absorbance readings were obtained for each well. Once the optimal concentrations of buffer pH and Triton X-100 were determined, the optimal concentrations of ATChI and DTNB for Ellman reagents were determined for the AChE assay using various combinations of ATChI and DTNB (0.1, 0.25, 0.5, 0.75, and 1.0 mM final concentrations for each reagent).

The AChE specific activity (U) (1U = conversion of 1 μM ATChI–DTNB in 1 min) was calculated as described by Srigiriraju et al. (2010).

$$U = \frac{(\text{mOD min}^{-1}\,1000^{-1}) \times 1000\ \text{mM min}^{-1}\,\text{mL}^{-1} \times \text{dilution factor}}{\text{Extinction coefficient of DTNB} \times \text{path length} \times \text{protein concentration}}$$

where the extinction coefficient of DTNB = 1.36×10^4 M^{-1} cm^{-1} and the path length is 0.6 cm.

AChE activity was determined in various life stages of *D. citri*. Five individuals of each life stage (third and fifth instars, or adults) and of each sex (adults only) were collected from the Lab strain. Enzymatic activity was estimated as described above at optimal conditions. Each treatment was replicated 3–8 times. In addition, AChE activity was determined in individual *D. citri* adults collected from the Lab strain or field populations. The procedure for extraction of AChE source from individual adults was similar to that described earlier with slight modifications. Each individual was homogenized in 100 μl of homogenization buffer with 0.1% Triton X-100 and centrifuged at 10,000 × *g* for 15 min at 4°C. After centrifugation, 90 μl of the supernatant was transferred into a new 1.5 ml centrifuge tube and diluted two-fold by adding 90 μl of homogenization buffer without Triton X-100. The AChE specific activity was determined as per the procedure described above.

Sensitivity of AChE to Inhibition by OPs and Carbamates. The extraction of enzyme source was similar to that described above using five adults per population. The assay was also similar to that described above with few modifications. Ten microliters of the enzyme extract (0.05 insect equivalent) was incubated with 10 μl of a serially diluted test insecticide (8–9 concentrations) for 2 min by shaking on a plate shaker. Each insecticide concentration was replicated three times and 10 μl of 0.1 M buffer substituted for insecticide served as the control. After incubation, 180 μl of Ellman reagents were added and the plate was read immediately at 405 nm and 25°C for 30 min at 20 s intervals in a microplate reader. Percent remaining AChE activity for each insecticide and *D. citri* population combination was calculated with respect to the activity in controls.

Activity of GEST in Adult *D. citri*. The activity of GEST in Lab strain and field-collected adults was measured using α-NA, β-NA, *p*-NPA, and *p*-NPB as substrates (Zhu et al. 2000, Wu et al. 2007). For the α-NA and β-NA substrates, the reaction mixture in each microplate well was comprised of 15 μl of enzyme extract and 135 μl of 0.3 mM α-NA or β-NA prepared in 0.1 M phosphate buffer (pH 7.5) giving a final concentration of 0.27 mM. Wells containing 15 μl of 0.1 M phosphate buffer as a substitute for enzyme preparation served as the control. Each treatment was replicated four to eight times. The plate was covered with aluminum foil and incubated at 37°C for 30 min. The reaction was stopped by adding 50 μl of fast blue B-sodium dodecyl sulfate solution. After 15 min at room temperature, the activity of GEST was measured in a microplate reader at 595 nm for α-NA and 560 nm for β-NA and 25°C. The specific activity of GEST was determined in nanometers of naphthol min^{-1} mg of $protein^{-1}$ using α-naphthol and β-naphthol standard curves, respectively, for α-NA and β-NA. The assay was repeated two to six times for each population of *D. citri*.

For *p*-NPA and *p*-NPB substrates, the reaction mixture in each microplate well consisted of 15 μl of the enzyme extract and 185 μl of 0.27 mM *p*-NPA or *p*-NPB prepared in 0.1 M phosphate buffer (pH 7.5) giving a final concentration of 0.25 mM. Wells containing 15 μl of 0.1 M phosphate buffer as a substitute for enzyme preparation served as the control. Each treatment was replicated four to eight times. The activity of GEST was measured immediately in a microplate reader at 405 nm and 27°C for 2 min. Using the molar extinction coefficient of 16.24 mM^{-1} cm^{-1}, the specific activity of GEST was determined as micromole min^{-1} milligrams of $protein^{-1}$. The assay was repeated two to five times for each population of *D. citri*.

Activity of GST in Adult *D. citri*. The activity of GST was measured using CDNB as the substrate (Wu et al. 2007, Zhu et al. 2000). The reaction mixture in each well consisted of 10 μl of the enzyme extract and 190 μl of CDNB–GSH solution [10.35 mM GSH and 200 mM CDNB in 188:2 (vol:vol) ratio]. Each plate was read immediately after preparation in a microplate reader at 340 nm and 25°C for 1 min. Ten microliters of phosphate buffer as a substitute for the enzyme extract served as the control. Each treatment was replicated four to eight times. Using the extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB, the specific activity of GST was determined as nanomoles min^{-1} milligrams of $protein^{-1}$. The entire assay was repeated two to six times for each population.

Protein Concentration. The protein concentration (milligrams per milliliter) for each enzyme source sample was determined by the bicinchoninic acid method using bovine serum albumin as the protein standard (Smith et al. 1985). The absorbance of the reaction product was measured in a microplate reader at 562 nm and 25°C.

Statistical Analyses. The concentration required for 50% inhibition of AChE activity (I_{50}) with 95% confidence intervals (CIs) was determined from concentration-percent remaining AChE activity data. These data were subjected to probit analysis to estimate I_{50} activity in *D. citri* adults using PROC PROBIT in SAS (SAS Institute 2005). Separate two-way analyses of variance (ANOVA) were performed to determine significance of main effects and their interactions for combinations of pH and Triton X-100 concentration, and for combinations of DTNB and ATChI using PROC GLM in SAS (SAS Institute 2005). Subsequent one-way ANOVAs were performed as needed and were followed by least significant difference (LSD) tests at $\alpha = 0.05$ (unprotected LSD). The mean specific activity of AChE and the total protein concentration in *D. citri* nymphs and adults from the Lab strain ($n = 7–15$), and mean specific activity of AChE, GEST, GST, and protein concentration among *D. citri* populations (Lab strain and field) ($n = 44–88$) were compared by one-way ANOVA followed by LSD tests.

Results

AChE Activity. The main effects of buffer pH and Triton X-100 concentration as well as the interaction

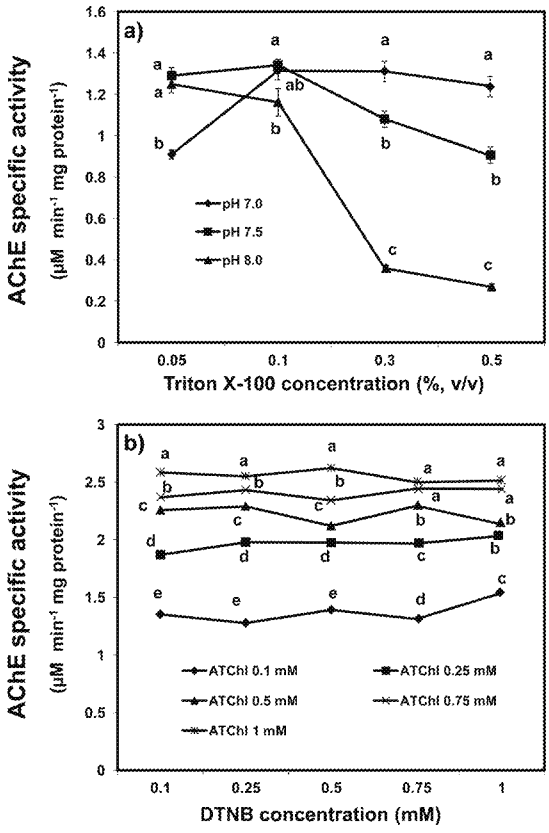

Fig. 1. Effect of (a) Triton X-100 concentration and phosphate buffer pH, and (b) DTNB and ATChI concentrations on specific activity of AChE. Each symbol represents mean ± SEM (n = 4–13). Symbols labeled with a different letter within a given concentration of Triton X-100 or DTNB are significantly different from one another according to LSD ($P < 0.05$).

between main effects (F = 69.31; df = 6, 144; P = <0.0001) significantly affected AChE specific activity. Although pH 7.5 + 0.1% Triton X-100 and pH 7.0 + 0.1% Triton X-100 combinations yielded statistically equivalent specific activities, the former combination was selected for further investigation because of its numerically higher activity (Fig. 1a).

The main effect of ATChI as well as the interaction between main effects (ATChI and DTNB concentrations) (F = 89.19; df = 16, 75; P = <0.0001) significantly affected AChE specific activity. A significant concentration-dependent increase in specific activity of AChE was observed with ATChI at all DTNB concentrations evaluated (Fig. 1b). Overall, a combination of 0.5 mM DTNB and 1.0 mM ATChI (final concentrations obtained by mixing 148 μl of 37.5 mM ATChI and 231.25 μl of 12 mM DTNB in 4620.75 μl of 0.1 M phosphate buffer pH 7.5) gave the highest AChE specific activity. This ratio was selected for further investigation.

The specific activity of AChE in *D. citri* nymphs and adults ranged from 1.23 to 2.21 μM min^{-1} mg of $protein^{-1}$. Among the various life stages evaluated, third

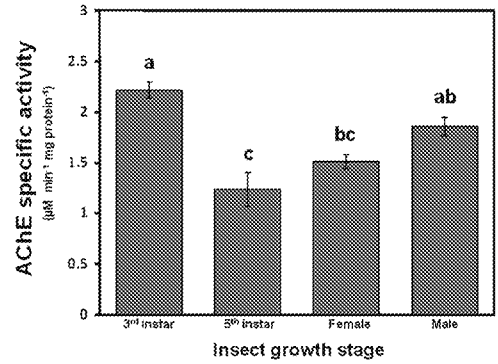

Fig. 2. Effect of *D. citri* growth stage on specific activity of AChE. Each bar represents mean ± SEM (n = 7–15). Bars labeled with different letters are significantly different from one another according to LSD ($P < 0.05$).

and fifth instars exhibited the highest (2.21 μM min^{-1} mg of $protein^{-1}$) and lowest (1.23 μM min^{-1} mg of $protein^{-1}$) AChE specific activities, respectively (F = 8.68; df = 3, 48; P = 0.0001) (Fig. 2). Both female and male adults exhibited statistically equivalent AChE specific activity (1.51 and 1.85 μM min^{-1} mg of $protein^{-1}$, respectively) (Fig. 2). With respect to the protein concentration, third instar nymphs were characterized by the lowest concentration (F = 18.09; df = 3, 48; P = <0.0001).

The AChE specific activity ranged from 0.32 to 2.47 μM min^{-1} mg of $protein^{-1}$. The AChE specific activity in field-collected adults was between 0.01 and 2.0 μM min^{-1} mg of $protein^{-1}$ (n = 44 for each field population) and that of the Lab strain adults was between 0.51 and 2.5 μM min^{-1} mg of $protein^{-1}$ (n = 88) (Fig. 3). The majority of adults (51%) from the Lab strain exhibited AChE activity between 1.01 and 1.5 μM min^{-1} mg of $protein^{-1}$ while 1% showed activity between 2.0 and 2.5 μM min^{-1} mg of $protein^{-1}$. In contrast, the majority of field-collected adults (77–

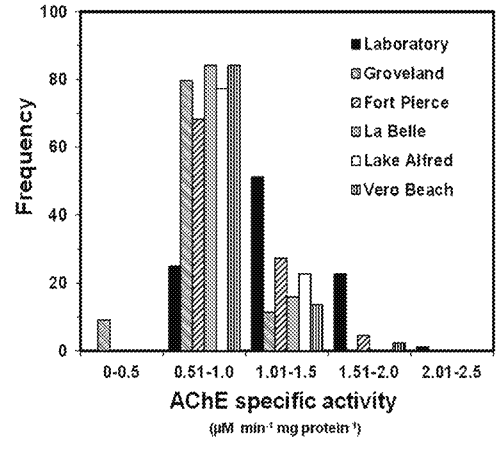

Fig. 3. Frequency distributions of *D. citri* adults (n = 44–88) from various populations with respect to specific activity of AChE.

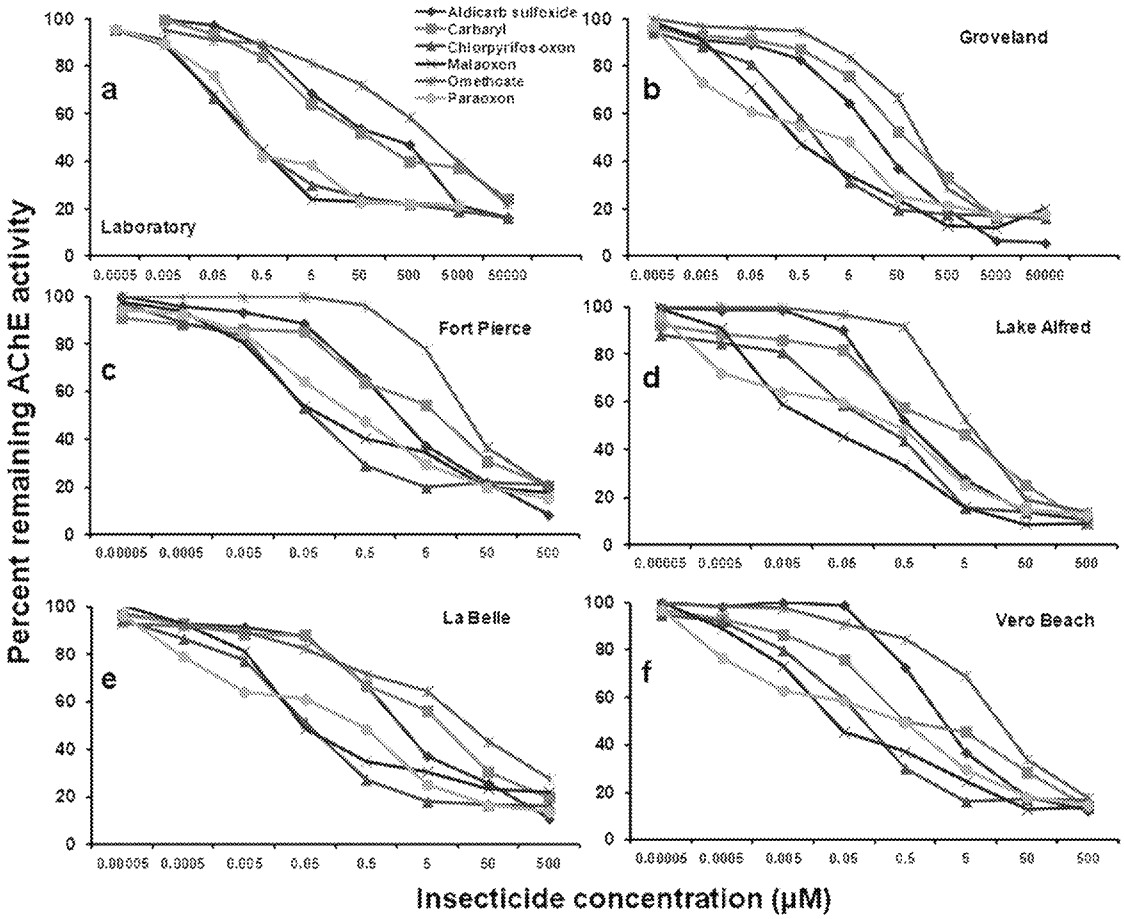

Fig. 4. AChE inhibitory potency curves for OP and carbamate insecticides in *D. citri* populations collected from (a) Lab strain, (b) Groveland, (c) Fort Pierce, (d) Lake Alfred, (e) La Belle, and (f) Vero Beach (n = 3 for each insecticide and population combination).

84%) exhibited activity between 0.51 and 1.0 μM min^{-1} mg of protein^{-1}. The next highest percentage of field-collected adults exhibited activity between 1.01 and 1.5 μM min^{-1} mg of protein^{-1}. Only adults collected from the Groveland site (9%) exhibited specific activity in the lower range of 0.01–0.5 μM min^{-1} mg of protein^{-1} (Fig. 3). The mean AChE specific activity was significantly higher in Lab strain than field-collected *D. citri* (F = 35.10; df = 5, 302; P = <0.0001).

The protein concentration in the Lab strain and in the field-collected adults ranged from 0.158 to 0.758 mg ml^{-1}. With the exception of Groveland *D. citri*, the majority of adults from the Lab strain (65%), Fort Pierce (59%), La Belle (52%), Lake Alfred (82%), and Vero Beach (64%) populations were characterized by protein concentrations ranging between 0.21 and 0.40 mg ml^{-1} followed by those ranging between 0.41 and 0.60 mg ml^{-1}. The opposite trend was found with the Groveland population, where the majority of adults (66%) exhibited a protein concentration in the range between 0.41 and 0.60 mg ml^{-1} with the next highest percentage ranging between 0.21 and 0.40 mg ml^{-1}. Only a small percentage of adults from the Lab strain (1%), Vero Beach (9%), and Groveland (14%) populations were in the lowest and highest protein concentration ranges. The Groveland population exhibited the highest protein concentration of those tested (F = 17.79; df = 5, 302; P = <0.0001).

Sensitivity of AChE to Inhibition by OP and Carbamate Insecticides. The comparison of inhibitory potency curves (concentration-% AChE activity remaining) of six insecticides for each *D. citri* population is given in Fig. 4a–f, while the comparison of inhibitory potency (I_{50} values) of each insecticide for six *D. citri* populations is presented in Table 1. AChE activity inhibition by each insecticide varied among the *D. citri* populations surveyed. In addition, a lag in inhibition was observed at the lower concentrations for omethoate (toxic form of dimethoate), aldricarb sulfoxide, (toxic form of aldicarb), and carbaryl for the field populations tested (Fig. 4a–f). Of the five insecticides tested, omethoate was least potent in inhibiting AChE activity followed by aldicarb sulfoxide and carbaryl compared with the standard inhibitor, paraoxon, (toxic form of parathion) (Table 1). With some ex-

Table 1. AChE inhibitory potency of OP and carbamate insecticides in various populations of *D. citri*

Insecticide	*D. citri* population	I_{50} (95% CIs)[a,b]	Slope ± SE	χ^2(df)	*P* value
Chlorpyrifos-oxon	Lab strain	0.14 (0.02–1.04)	−0.36 ± 0.05	34.68 (6)	<0.0001
	Groveland	0.28 (0.04–1.79)	−0.35 ± 0.05	41.04 (7)	<0.0001
	Fort Pierce	0.38 (0.03–4.00)	−0.34 ± 0.06	59.57 (7)	<0.0001
	La Belle	0.17 (0.01–1.25)	−0.35 ± 0.05	47.02 (7)	<0.0001
	Lake Alfred	0.21 (0.03–1.15)	−0.36 ± 0.05	27.88 (6)	<0.0001
	Vero Beach	0.27 (0.03–1.85)	−0.37 ± 0.05	47.88 (7)	<0.0001
Malaoxon	Lab strain	0.12 (0.01–1.23)	−0.35 ± 0.06	43.71 (6)	<0.0001
	Groveland	0.13 (0.03–0.48)	−0.44 ± 0.04	20.58 (6)	0.002
	Fort Pierce	0.45 (0.13–1.69)	−0.40 ± 0.04	18.13 (6)	0.005
	La Belle	0.34 (0.02–3.44)	−0.35 ± 0.06	29.73 (5)	<0.0001
	Lake Alfred	0.06 (0.01–0.27)	−0.47 ± 0.06	27.44 (6)	0.0001
	Vero Beach	0.17 (0.02–0.44)	−0.39 ± 0.04	13.65 (5)	0.01
Omethoate	Lab strain	102.40 (50.24–231.82)	−0.35 ± 0.02	5.63 (6)	0.46
	Groveland	123.72 (38.50–523.04)	−0.57 ± 0.07	22.72 (6)	0.0009
	Fort Pierce	358.46 (247.51–530.17)*	−0.89 ± 0.07	5.67 (6)	0.46
	La Belle	216.82 (94.81–582.42)	−0.31 ± 0.02	7.29 (6)	0.29
	Lake Alfred	105.77 (48.74–228.62)	−0.76 ± 0.07	18.17 (7)	0.01
	Vero Beach	164.38 (99.09–287.03)	−0.55 ± 0.03	9.91 (6)	0.12
Paraoxon	Lab strain	0.21 (0.03–1.60)	−0.36 ± 0.05	34.77 (6)	<0.0001
	Groveland	0.15 (0.03–0.71)	−0.32 ± 0.04	18.01 (6)	0.006
	Fort Pierce	0.55 (0.32–0.99)	−0.42 ± 0.02	6.28 (6)	0.39
	La Belle	0.18 (0.06–0.56)	−0.36 ± 0.03	11.85 (6)	0.06
	Lake Alfred	0.13 (0.03–0.49)	−0.35 ± 0.03	15.82 (6)	0.01
	Vero Beach	0.19 (0.05–0.65)	−0.34 ± 0.03	12.84 (6)	0.04
Aldicarb-sulfoxide	Lab strain	16.55 (9.79–28.70)	−0.46 ± 0.02	10.06 (6)	0.12
	Groveland	13.34 (8.14–22.03)	−0.48 ± 0.02	11.96 (7)	0.10
	Fort Pierce	25.56 (16.08–40.81)	−0.54 ± 0.02	11.84 (7)	0.10
	La Belle	23.78 (10.27–56.64)	−0.47 ± 0.03	13.24 (7)	0.06
	Lake Alfred	16.27 (5.56–46.86)	−0.62 ± 0.02	27.83 (7)	0.0002
	Vero Beach	47.61 (15.09–149.10)	−0.6 ± 0.08	33.22 (7)	<0.0001
Carbaryl	Lab strain	21.70 (6.42–89.72)	−0.36 ± 0.03	14.58 (6)	0.02
	Groveland	64.48 (21.70–240.76)	−0.42 ± 0.04	13.17 (6)	0.04
	Fort Pierce	46.10 (12.12–250.11)	−0.33 ± 0.03	14.65 (6)	0.02
	La Belle	60.88 (21.23–82.79)	−0.40 ± 0.02	8.52 (6)	0.20
	Lake Alfred	13.01 (4.62–38.25)	−0.41 ± 0.03	16.90 (7)	0.01
	Vero Beach	16.10 (9.10–29.50)	−0.41 ± 0.02	4.32 (6)	0.63

[a] Concentration in micromoles for 50% AChE activity inhibition.
[b] 95% CIs for I_{50}.
*Significantly different from Lab strain based on nonoverlap of 95% CIs.

ceptions, chlorpyrifos-oxon and malaoxon (toxic forms of chlorpyrifos and malathion, respectively) exhibited comparable inhibitory potency to paraoxon in all *D. citri* populations tested. The overlap of 95% CIs of I_{50} values revealed no significant differences in AChE sensitivity to the OP and carbamate insecticides tested between the Lab strain and each field population or among the field populations with the exception of a difference between the Lab strain and the Fort Pierce population for omethoate (Table 1).

Specific Activity of GEST in Adult *D. citri*. Specific activity of GEST in various populations ranged from 249.24 to 410.46 nM min^{-1} mg of $protein^{-1}$ with α-NA; 23.71–53.41 nM min^{-1} mg of $protein^{-1}$ with β-NA; 29.03–50.11 μM min^{-1} mg of $protein^{-1}$ with *p*-NPA; and 27.95–36.08 μM min^{-1} mg of $protein^{-1}$ with *p*-

Table 2. Specific activity of AChE, GEST, and GST in various populations of *D. citri*

D. citri population	AChE	GEST				GST
	ATChI[a]	α-NA[b]	β-NA[b]	*p*-NPA[c]	*p*-NPB[c]	CDNB[c]
Lab strain	1.29 ± 0.03a	249.24 ± 50.42a	23.71 ± 4.93c	29.03 ± 3.52b	27.95 ± 1.44a	159.25 ± 18.79b
Groveland	0.77 ± 0.03d	410.46 ± 18.75a	53.41 ± 11.25a	50.11 ± 13.69a	36.08 ± 8.13a	222.37 ± 6.43ab
Fort Pierce	1.00 ± 0.03b	303.13 ± 52.61a	31.88 ± 3.45bc	32.60 ± 1.85ab	32.21 ± 3.79a	286.70 ± 21.40a
La Belle	0.87 ± 0.02cd	291.44 ± 32.27a	38.56 ± 5.15ab	35.91 ± 3.50ab	30.39 ± 4.62a	218.91 ± 30.09ab
Lake Alfred	0.93 ± 0.03bc	364.19 ± 43.93a	30.05 ± 1.05bc	34.54 ± 6.53ab	29.95 ± 4.02a	204.91 ± 22.88ab
Vero Beach	0.95 ± 0.02bc	273.08 ± 30.95a	31.80 ± 5.42bc	40.60 ± 6.13ab	31.21 ± 3.56a	223.63 ± 39.93ab

[a] μM min^{-1} mg of $protein^{-1}$.
[b] nM min^{-1} mg of $protein^{-1}$.
[c] μM min^{-1} mg of $protein^{-1}$.
Means (± SEM; n = 2–88) followed by different letters in a column are significantly different from one another according to LSD. $P < 0.05$.

NPB (Table 2). There were no significant differences in the specific activities of GEST among the *D. citri* populations tested when α-NA and *p*-NPB were used as substrates (Table 2). However, significant differences were found in enzyme activity when β-NA and *p*-NPA were used as substrates (Table 2). The Groveland population and the Lab strain exhibited the highest and lowest specific activities, respectively, which were statistically different from one another (Table 2).

Specific Activity of GST in Adult *D. citri*. Specific activity of GST in various populations ranged from 159.25 to 286.70 μM min^{-1} mg of protein^{-1} with CDNB as a substrate (Table 2). The Fort Pierce population and the Lab strain exhibited the highest and lowest specific activities, respectively, which were statistically different from one another but not from the remaining populations (Table 2).

Discussion

Rotation of OP and carbamate insecticides with different classes and modes of action such as neonicotinoids, IGRs, and insect antifeedants (Boina et al. 2010 and 2011, Tiwari et al. 2011a) should mitigate resistance development to these chemistries in *D. citri*. One of the mechanisms conferring resistance to OP and carbamate insecticides is reduced sensitivity of AChE (Gao and Zhu 2000, Byrne et al. 2003). Although increased activity of detoxifying enzymes such as GEST and GST alone may contribute to resistance development (Tiwari et al. 2011a and b), concurrent emergence of target site insensitivity will further facilitate potential insecticide failures (Srigiriraju et al. 2010). The purpose of this investigation was to establish baseline activity of AChE and its sensitivity to selected OP and carbamate insecticides among Florida populations of *D. citri*. We also concurrently determined levels of GEST and GST enzymes among these same populations of *D. citri*.

Initially, we optimized the bioassay conditions for accurate quantification of AChE activity. Because phosphate buffer pH and concentration of Triton X-100 influence the release of AChE from insect tissues and concentration of Ellman reagents (DTNB and ACThI) affects the enzyme reaction, the AChE assay conditions required optimization for *D. citri*. It appears that an increase in Triton X-100 concentration above 0.1% interfered with the enzyme reaction. Irrespective of buffer pH, a Triton X-100 concentration >0.1% decreased AChE activity (Fig. 1a). Concentrations of ATChI and DTNB up to 1 mM did not adversely affect AChE activity.

Quantifying AChE specific activity and protein concentration in immature and adult *D. citri* revealed that third instars exhibited the highest AChE specific activity among the life stages evaluated and this may be attributed to lower protein content in third instars than in other life stages. Similarly, the higher protein concentration in females than males resulted in lower AChE specific activity in the former. This relationship suggests that amount of protein may be an important characteristic impacting evolution of resistance. It appears that binding of insecticides to cellular macromolecules, mainly proteins, results in reduced active quantities available at the target site (Hollingsworth 1986, Devonshire and Moores 1982). Greater insecticide susceptibility of early instar *D. citri* compared with adults (L.L.S., unpublished data) may be explained by lower amounts of protein in the former. This hypothesis is congruent with previous results; workers of a Formosan subterranean termite, *Coptotermes formosanus* Shiraki, were characterized by higher protein content than soldiers, and were less susceptible to chlorpyrifos than soldiers (Gatti et al. 2002).

Given that specific activity of AChE from the Lab strain predominantly overlapped with that of the field populations suggests that the AChE molecules from the various populations evaluated were closely related. AChE from the Lab strain was highly sensitive to inhibition by the OP and carbamate insecticide evaluated, while AChE from field-collected populations showed slight insensitivity (a lag in AChE activity inhibition) to three of the six insecticides at lower concentrations. The current results indicate no significant reduction in AChE sensitivity to OP and carbamate insecticides in Florida populations of *D. citri*.

Of the six insecticides investigated here, five were tested using their active form with in vitro assays. Because their active form is readily available for interaction with the target site in vitro, the inhibitory potency of these insecticides depended upon their binding affinity to AChE. However, under field conditions (or in toxicity bioassays), these insecticides are bioactivated in vivo into their toxic form (except carbaryl) by insect cytochrome P_{450} monooxygenases or detoxified by GEST and GST. Therefore, inhibitory potency of these insecticides in the field depends on the availability of the active form of these compounds at the target site, which depended on the rate of bioactivation and metabolic detoxification as well as on binding affinity with the target site. In addition to the rate of bioactivation, other resistance mechanisms such as reduced cuticular penetration could account for higher LD_{50} resistance ratios reported previously (Tiwari et al. 2011a).

Of the three OP insecticides tested, omethaote was the least potent inhibitor of AChE in *D. citri* requiring 102.40–358.46 μM for 50% inhibition of enzyme activity. Despite the low inhibitory potency of omethoate, its stability and potential for accumulation at the target site results in similar anti-acetylcholinesterase activity as compared with other OP insecticides at substantially lower concentrations (Devonshire et al. 1975). Omethoate is known to be a poor inhibitor of AChE in glassy-winged sharpshooter, *Homalodisca vitripennis* (Germar) (328 μM), and smoke-tree sharpshooter, *H. lacerta* Fowler (299 μM), as compared with chlorpyrifos-oxon and paraoxon (Byrne et al. 2003).

The consistent trend for lower specific activities of GEST and GST in the Lab strain than in field popu-

lations is indicative of lack of exposure to selection pressure or influx of resistant genes from field populations into the Lab strain. Overall, the activity levels of detoxifying enzymes in all field populations tested were higher than in the Lab strain that may partly explain the higher resistance ratios reported for bifenthrin, chlorpyrifos, fenpropathrin, imidaclorpid, and thiamethoxam in field populations (Tiwari et al. 2011a). Use of α-NA and p-NPB as substrates to GEST did not reveal statistical differences in GEST activities among the populations tested, while use of β-NA and p-NPA as substrates yielded significant differences. However, GEST levels were higher in the same field-collected populations than in the Lab strain using α-NA as the substrate in 2010 (Tiwari et al. 2011a), suggesting a progressive shift in resistance levels as a function of detoxifying enzymes after only 1 yr. Differential level of GEST activities as a result of different substrates suggests presence of isoenzymes in the tested *D. citri* populations that prefer one substrate over the other.

Insecticide resistance management should be implemented before the discovery of product failures in the field. Establishing baseline sensitivities allows monitoring of potential shifts given that optimal resistance management is not always practiced. Biochemical assays suggested that field populations of *D. citri* exhibited significantly reduced activity of AChE compared with a susceptible Lab strain. AchE insensitivity is likely the biochemical basis of organophosphate and carbamate resistance in *D. citri*; however, in some specific populations, such as Groveland, elevated activities of carboxylesterases and GSTs might also contribute toward resistance. The data presented here on sensitivity of AChE to inhibition and on specific activity of various detoxifying enzymes should provide an additional baseline to previously reported toxicological data (Tiwari et al. 2011a) for biochemical monitoring of OP and carbamate resistance in *D. citri*.

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